Survival Potential of *Escherichia coli* and Enterococci in Subtropical Beach Sand: Implications for Water Quality Managers

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Fecal bacteria have traditionally been used as indicator organisms to monitor the quality of recreational waters. Recent work has questioned the robustness of traditional indicators, particularly at seawater bathing beaches. For example, a study of Florida beaches found unexpectedly high abundances of *Escherichia coli*, fecal coliforms, and enterococci in beach sand. The aim of the present study was to explain these abundances by assessing the survival of *E. coli* and enterococci in beach sand relative to seawater. We used a combination of quantitative laboratory mesocosm experiments and field observations. Results suggested that *E. coli* and enteroccocci exhibited increased survivability and growth in sand relative to seawater. Because fecal bacteria are capable of replicating in sand, at least under controlled laboratory conditions, the results suggest that sand may be an important reservoir of metabolically active fecal organisms. Experiments with "natural" mesocosms (i.e., unsterilized sand or water rich in micropredators and native bacteria) failed to show the same increases in fecal indicators as was found in sterile sand. It is postulated that this was due to predation and competition with indigenous bacteria in these "natural" systems. Nonetheless, high populations of indicators were maintained and recovered from sand over the duration of the experiment as opposed to the die-off noted in water. Indicator bacteria may wash out of sand into shoreline waters during weather and tidal events, thereby decreasing the effectiveness of these indicators as predictors of health risk and complicating the interpretations for water quality managers.

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Bacterial indicators are used to assess the health risks associated with bathing in sewage-contaminated recreational waters. In 1986, the United States Environmental Protection Agency (USEPA) produced guidelines recommending enterococci and *Escherichia coli* as appropriate indicators to monitor recreational waters (USEPA, 1986). These guidelines were based on the findings that enterococci and *E. coli* had a high positive correlation with instances of swimming-associated gastroenteritis. Possible sources of these bacteria included raw human sewage, outfalls from sewage treatment facilities, and storm drain runoff containing feces of animals (Pommepuy et al., 1992; Roll and Fujioka, 1997). Even treated effluent from sewage treatment facilities can contain pathogenic microorganisms (Bitton et al., 1984) because bacteria can survive the final chlorination treatment as resistant spores or by being sheltered in the center of aggregated particles.

In a recent 2-yr study of three south Florida beaches, the levels of *E. coli*, enterococci, and fecal coliforms were on average 10 times higher in wet sand and 100 times higher in dry sand relative to shoreline water (Bonilla et al., 2006; 2007). The authors reported that dry sand averaged around 2.5×10^4 enterococci kg^{-1} of sand, although these bacteria were not evenly distributed in the sand. For example, enterococci could range from below detection to 4.9×10^5 cells kg^{-1} across 20 samples taken just 0.1 m apart. These high densities in the sand need to be explained because they question the robustness of currently used indicator systems. Moreover, if beach sand is a significant reservoir for indicator organisms, shoreline waters might receive substantial bacterial loads of environmental indicators during tidal cycles and weather events. Solo-Gabriele et al. (2000) found that high numbers of *E. coli* present in soil along

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Abbreviations: BHIB, brain-heart infusion broth; mEI agar, m-enterococcus agar plates with indoxyl-β-d-glucoside; PBS, phosphate-buffered saline; PSU, practical salinity units; USEPA, United States Environmental Protection Agency.

a river bank were washed into the water during tidal cycles, and Desmarais et al. (2002) demonstrated that tidally influenced soils harbor elevated populations of enteric bacteria.

There have been several reports showing that indicator organisms are capable of surviving and growing in the environment (Davies et al., 1995; Sussman, 1997; Desmarais et al., 2002). If true, this violates one of the criteria applied to indicators, namely that a fecal indicator must not grow in the natural environment (Dufour, 1984). *Escherichia coli*, fecal coliforms, and enterococci have been found in freshwater, soil, and plant samples removed from obvious human contamination (Hardina and Fujioka, 1991; Anderson et al., 1997; Fujioka, 2001; Desmarais et al., 2002), although this does not eliminate the possibility that contamination was introduced by animals. Bird feces, for example, are rich in fecal bacteria (Oshira and Fujioka, 1995).

Reports of high numbers of indicator bacteria in beach sand (Bonilla et al., 2007), their occurrence at locations remote from obvious sewage contamination, and the notion that these bacteria are growing in soils and sediments combine to potentially weaken the usefulness of enterococci and *E. coli* as indicators for monitoring recreational water quality. Understanding the dynamics of indicator populations on a beach is crucial if we are to retain confidence in these organisms as indicators of potential health risks to beach users. The high numbers in the sand could be a consequence of in situ growth, land runoff, or the filtering action of sand removing bacteria from the water during tidal cycles.

Several studies have suggested that enteric bacteria show increased survival in sand and sediments, although few studies focus on marine beaches. Most studies on this topic conclude that enteric bacteria exhibit increased survival in sand and sediment possibly because particulates provide microbes with a unique microhabitat affording protection, increased moisture, and nutrients (Gerba and McLeod, 1976; Pommepuy et al., 1992; Davies et al., 1995, Howell et al., 1996; Desmarais et al., 2002).

Filtration is also a factor that accounts for the high densities of indicator bacteria in sand and sediments. When sewage effluent was allowed to filter through a 3-ft column of soil, the number of fecal bacteria in the effluent was reduced by over 50% (Gerba et al., 1975). Likewise, Hartz (unpublished data) showed that a 1.4-m column of sterilized sand removed 65.6% of enterococci from a water sample allowed to percolate slowly through wet sand. When nonsterilized sand was used, the filter removed 92.0% of the bacteria, possibly due to matrix effects and/or predation by indigenous micrograzers.

The present study sought to find explanations for the high counts of *E. coli* and enterococci observed in Florida beach sand by conducting laboratory mesocosm experiments to compare bacterial survival in sand versus seawater. Although these batch culture systems were highly artificial, they did allow examination of single variables on bacterial survival, predominantly temperature and salinity levels reflecting those found on the beach. The study also considered whether fecal indicators were washed from the sand into the water during tidal and weather events and how these might complicate the interpretation of levels detected in the water during routine water quality monitoring.

Materials and Methods

Isolation of Fecal Indicator Bacteria

The bacterial isolates for the mesocosm experiments were isolated from Hollywood Beach, Florida (26°02'0.256"N, 80°06'50.2"W). For *E. coli* and enterococci, a total of six isolates were prepared: two from dry sand, two from wet sand, and two from shoreline water at a depth of 0.5 m. For comparative purposes, an additional six isolates of enterococci were recovered from fresh human feces (only enterococci were compared because these are the preferred indicators for marine waters). In all cases, phosphate-buffered saline (PBS) was added to the particulate material, and samples were shaken for 1 min in a sterile glass media bottle. Aliquots of supernatant containing the suspended bacteria were vacuum filtered (<10 mm Hg) on sterile 0.45-µm filters (nitrocellulose). Samples of raw seawater were also shaken for 1 min and vacuum filtered on identical filters. In the case of *E. coli*, filters were transferred onto membrane thermotolerant *E. coli* agar plates (selective media for *E. coli*; Difco; Becton Dickinson, Franklin Lakes, NJ) and incubated at 35 ± 0.5°C for 2 h then at 44.5 ± 0.2°C for 22 h. After incubation, filters were placed on sterile pads saturated with urea broth for 20 min for confirmation (AWWA, 1998). Six random colonies of *E. coli* (yellow or yellow-green colonies after confirmation) were inoculated onto tryptic soya agar (Difco; Becton Dickinson) and maintained by regular subculturing.

In the case of enterococci, filters were transferred onto m-enterococcus agar plates with indoxyl-β-d-glucoside (mEI agar) and incubated for 24 h at 41 ± 0.5°C. Colonies with blue halos are considered to be enterococcus The USEPA recommends an additional procedure to ensure that colonies are verified as enterococcus. Colonies were verified as enterococcus by transfering subsamples from six colonies with blue halos into brain-heart infusion broth (BHIB) tubes (24 h, 35°C) (Difco; Becton Dickinson) and onto a BHI agar slant (48 h, 35°C) (Difco; Becton Dickinson). After 24 h, bacteria from each BHIB tube were transferred to BHIB (48 h, 45°C), BHIB + 6.5% NaCl (48 h, 35°C), and Bile Esculin Agar plates (48 h, 35°C) (Difco; Becton Dickinson). Growth was verified from all these mediums, colonies on BEA plates produced a black/brown precipitate, and a gram stain was applied. Gram-positive cells were verified as enterococcus (USEPA, 2002). Isolates were maintained by regular subculturing on BHI agar (Difco; Becton Dickinson).

Preparation of the Inoculum

Two days before each experimental run, the bacterial isolates were subcultured to ensure healthy, exponentially growing cells for experimentation. Twenty-four hours before the experiment, the six strains of *E. coli* or enterococci were grown in a Petri dish on tryptic soya agar. On the day of the experiment, about 3 mL of sterile PBS was added to the dish, and the strains of bacteria were suspended in the PBS. The density of bacteria in this suspension was standardized with a calibrated turbidity meter (Cole Parmer, Vernon Hills, IL). Typically, enterococci were added to give a final count of 5×10^5 bacteria L^{-1} of water or liter of sand, and *E. coli* were

added to give a final count of 3×10^5 bacteria L^{-1} of water or liter of sand.

Seawater and Sand Mesocosms

All the mesocosm experiments were performed in triplicate in large (3 L), sterile, covered beakers. Sand mesocosms were prepared as follows. Intertidal sand was collected from Hollywood Beach, Florida. In the laboratory, the sand was rinsed several times to remove salt, oven dried at 105°C until constant weight, and autoclaved. The moisture content of the sand was adjusted to mimic the moisture of wet and dry sand by adding an appropriate volume of sterile seawater (wet sand: 560 mL per 3 L of sand; dry sand: 280 mL per 3 L of sand). The volume of water added was determined by weighing freshly collected wet and dry sand before and after oven drying. The seawater used for the sand mesocosms was collected from Hollywood Beach, Florida, vacuum filtered through a glass fiber filter, and autoclaved. The water salinity was adjusted by adding sea salts (Sigma Chemical Co., St. Louis, MO) to elevate the salinity or sterile distilled water to decrease salinity. Before adding the seawater to the sand, *E. coli* or enterococci were added to give the densities (per L) stated previously. This bacterial suspension was mixed thoroughly before adding to the sand.

For all seawater mesocosm experiments, seawater was collected from Hollywood Beach, Florida, and salinity was adjusted by adding sea salts or distilled water (as was done for the sand mesocosms). The seawater was autoclaved, and the experimental bacteria were added to the water and mixed thoroughly. Methods development for these experiments included testing the sterility of sand and seawater before inoculation with fecal bacteria; the sand and seawater were free of living bacteria preceding inoculation. For both mesocosms (sand and water), the survival of fecal bacteria was monitored over time (typically 2 wk).

The survival of *E. coli* and enterococci was monitored in sand and seawater at temperatures of 20, 30, and 40°C (at a salinity of 32 practical salinity units [PSU]); subsequent experiments were performed at 30°C for *E. coli* and 20°C for enterococci based on optimal survival from temperature experiments. Similarly, the effects of salinity (6, 15, 32, and 38.5 PSU for *E. coli* and 6, 15, 32, 40 PSU for enteroccocci) on bacterial survival were examined. Survival was examined in wet sand versus dry sand at 30°C for *E. coli* and 20°C for enterococci.

One experiment also considered nutrient enrichment of sand and water to determine whether the presence of added nutrients affected bacterial survival. In this case, sand and water mesocosms received 1 or 20 mL of a concentrated soil extract prepared by autoclaving 0.1 kg of garden loam soil (garden top soil from Home Depot, Dania Beach, FL) in 0.5 L of seawater for 1 h. After settling and vacuum filtering through a glass fiber filter, the extract was re-autoclaved and frozen until needed. These mesocosms were inoculated with the same densities of *E. coli* and enterococci as used in the sterile sand or seawater mesocosms. Experiments were conducted at a salinity of 32 PSU and a temperature of 30°C (*E. coli*) or 20°C (enterococci).

For both fecal indicator types, "natural" mesocosms were set up using freshly collected wet sand and water. These were not autoclaved so that live indigenous bacteria and micro- and macropredators were present. The number of fecal bacteria in the sand was counted before adding inocula. In this way, a correct total bacterial count was attained at time 0. The salinity was ambient (approximately 32 PSU), and incubations were at 30°C in the case of the *E. coli* mesocosms and 20°C in the case of enterococci.

The survival of the six isolates of enterococci recovered from human feces was monitored after inoculating into sand and water at 20°C with a salinity of 32 PSU. This was to determine whether recently isolated fecal bacteria survived differently from indicator isolates from the beach environment. Experiments were conducted as described for previous sand and water mesocosms.

Sampling of Mesocosms

Bacteria were sampled daily for 2 wk or until bacterial survival was 5% or less of the starting density. This cut-off level was chosen because it represents 95% kill—a level often used in toxicity testing—and represents substantial die-off. For the sand mesocosms, 0.1 kg of sand was sampled by removing a core from the surface of the sand to the bottom of the beaker using a large sterile spatula. The sample was placed in a sterile media bottle, PBS was added, and the bacteria were removed from sand by vigorously hand shaking for 1 min. Several replicate aliquots of PBS with suspended bacteria ($n = 3$) were filtered to ensure a count of discrete colonies on the filter. The volume varied depending on whether bacteria increased or decreased and was based on the density 24 h prior. Aliquots were filtered onto 0.45-µm filters (mixed cellulose ester; Pall Corporation, Ann Arbor, MI), which were placed on the same medium used in their isolation. For the water mesocosm, the contents of the beaker were thoroughly agitated with a sterile pipette to disrupt flocs, dislodge any attached cells, and resuspend settled bacteria. Subsequently, 0.1 L of water was sampled and shaken for 1 min. Three replicate aliquots of water were vacuum filtered onto a 0.45-µm filter. After incubation, the numbers of cells L^{-1} (colony-forming units) were determined.

Attached versus Unattached Bacteria

Experiments were conducted on wet and dry beach sand collected from Hollywood Beach to determine the relative percentage of attached versus interstitial (free) enterococci and fecal coliforms in the sand.

A 0.2-kg sample of freshly collected wet beach sand was placed in a cylinder (0.1 m in diameter, 0.1 m in height) with 80 µm mesh on the base to contain the sand. The cylinder was gently dipped into 0.2 L of sterile PBS 10 times to wash away free interstitial bacteria. The PBS containing bacteria was filtered through a 0.45-µm Millipore filter to collect bacteria present in the washings. The bacteria were incubated on the appropriate media: mEI for enterococci and membrane fecal coliform agar (Difco; Becton Dickinson) for total fecal coliforms. An additional 0.2-kg sample of sand was shaken vigorously with 0.2 L of sterile PBS for 1 min to remove interstitial and attached bacteria. The PBS with suspended bacteria was filtered through a 0.45-µm filter, and bacteria were incubated on the appropriate media. Bacteria were enumerated, and the relative percentage of attached and interstitial bacteria in freshly collected wet and dry beach sand

was calculated. Wet sand was collected from the intertidal zone at low tide, and dry sand was collected from approximately 3 m above the high tide line. This experiment was repeated five times.

Direct Observation of Attached *E. coli*

A laboratory strain of *E. coli* (strain JM109) transformed with a green fluorescent protein (GFP) expression plasmid (BD Bioscience Clontech, Palo Alto, CA) was supplied by one of the authors (N. Esiobu, Florida Atlantic University). When *E. coli* containing the GFP are examined under UV light, the cells fluoresce bright green. Green fluorescent proteins expressing *E. coli* were used to directly observe the attachment of fecal bacteria on sand grains. The GFP-expressing *E. coli* was lifted from an agar slant with an inoculating loop and mixed with 10 mL of sterile seawater in a 15-mL conical tube. The tube was shaken until the *E. coli* were evenly dispersed. A 1-mL aliquot of the suspension (aproximately 10^7 bacteria) was added to 50 mL of sterile seawater containing approximately 10 sterile sand grains. The beaker was stored at room temperature, and after 24 and 48 h sterile forceps were used to retrieve individual sand grains. These were washed thoroughly by repeatedly dipping them into a beaker of sterile seawater to remove loose surface bacteria from the sand grain. Sand grains were placed on a glass cover slip and viewed with an inverted light microscope at 900× magnification by epifluorescent microscopy (excitation: approximately 380 nm; emission: approximately 500 nm). A second experiment was conducted with separate beakers containing silica and calcium carbonate sand grains to determine if either type of sand promoted increased attachment of *E. coli*.

Bacterial Counts in the Swash Zone and up to 3000 m Offshore

To document the potential for shoreline samples to be influenced by indicator bacteria in the sand (washing out during tidal or weather effects), samples of seawater were collected from 0.1 m to 3000 m offshore. Inshore samples (0.1–20.0 m) were collected from Hollywood Beach, Florida between October 2002 and August 2003. On each sampling event, water was collected in triplicates in sterile media bottles (or in 50-mL conical tubes for the shallowest samples) at 0.1, 1.0, 3.0, 10.0, and 20.0 m from the shore line. Samples were collected during a wide range of weather, wind, and wave conditions and during incoming and outgoing tides. All samples were returned on ice to the laboratory and processed within 2 h. For each sample collected, 0.1-L aliquots were vacuum filtered through a 0.45-µm filter and transferred onto membrane thermotolerant *E. coli* or mEI for *E. coli* or enterococci, respectively.

Over the same timeframe, offshore seawater samples (n = 717) were collected from water between 200 and 3000 m off Hollywood Beach, Florida. For each location, two replicate surface samples (0.3 m in depth) were collected and placed on ice and processed within 6 h of collection. Upon return to the laboratory, 0.2 L of seawater was filtered from each sample onto a 0.45-µm filter. In some cases (n = 67), deeper samples (4.5–9.0 m in depth) were collected by SCUBA divers. The location of samples was determined by GPS, and the entire offshore beach area was randomly sampled, including upper water column areas close to the sewage outfall pipe from the Hollywood sewage treatment plant and outflows from the Port Everglades channel. For each sample, the membrane filtration method was used for the detection of enterococci (USEPA Method 1600) using mEI agar as described previously.

Table 1. Number of cell doublings (i.e., generations) of fecal bacteria after 4 d in sand and water mesocosm experiments. Unless otherwise specified, the temperature used for the *Escherichia coli* mesocosms was 30°C and 20°C for enterococci. In both cases the standard salinity was 32 practical salinity units (PSU).

	E. coli		Enterococci	
	Sand	Water	Sand	Water
Temperature, °C	14	1	9	0†
20				
30	18‡	0	4	0†
40	9	0†	4	0†
Salinity, PSU				
6	10	0	7§	0¶
13	15	0#	4§	0††
32	18	1	9§	0†
38.5	15	0†	6§	0††
Nutrients				
1 ml soil extr.	12	1	7	0††
20 ml soil extr.	12	1	7	0††
Moisture content				
Dry sand	11	–	9	–
Wet sand	11	–	9	–
Natural dry sand‡‡		–	0	–
Natural wet sand‡‡	0§§	–	0	–
Isolate type				
Fresh¶¶	–	–	6	0

† By Day 4, survival was <5%.

‡ Density after 4 d peaked at 7.3×10^8 bacteria kg^{-1}.

§ Numbers remained significantly ($p < 0.05$) above starting concentrations over 14 d of treatment.

¶ Significant decrease ($p < 0.05$) in density by Day 7.

By Day 13, densities were significantly lower ($p = 0.02$) than starting concentration.

†† No significant ($p < 0.05$) decrease over duration of experiment (14 d).

‡‡ Natural wet sand was not sterilized and contained indigenous bacteria and micro-grazers.

§§ By Day 2, survival was <5%.

¶¶ Freshly isolated fecal enterococci.

Results

The influence of physical and chemical conditions on the proliferation and persistence of enterococci and *E. coli* in beach sand and water was investigated using artificial mesocosm experiments. Table 1 summarizes the data obtained from the experiments. Parameters considered in this study included salinity, temperature, nutrients, water content, and competition/predation from indigenous sand microorganisms. In all cases (except the natural sand mesocosms), bacteria underwent several divisions in sand (between 4 and 18 generations), compared with between 0 and 1 division in water. These doubling events in sand represent sustained growth, taking populations to densities as high as 7.3×10^8 bacteria kg^{-1} of sand.

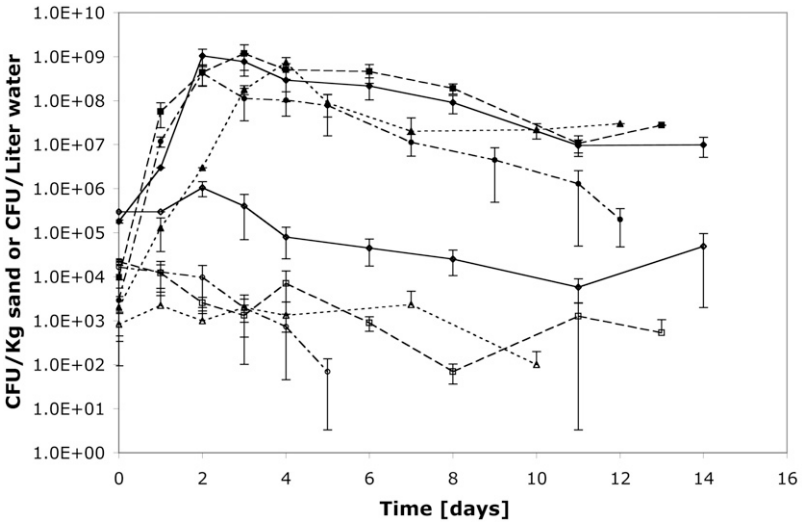

Fig. 1. Survival of *Escherichia coli* in sterile beach sand and water (colony-forming units [CFU] kg^{-1} sand or CFU L^{-1}) over a range of salinities at 30°C. Solid symbols represent sand; open symbols represent water. Shapes correspond to salinity: diamond, 6 practical salinity units [PSU]; square, 15 PSU; triangle, 32 PSU; circle, 38.5 PSU. Error bars represent 1 SE (n = 3).

A representative data set is shown in Fig. 1 and 2 to illustrate the trends generally found during the different treatments. Here, the survival of enterococci (Fig. 2) at 20, 30, and 40°C in wet sand adjusted to a salinity of 32 PSU was compared with survival in seawater under the same conditions. In the sand, enterococci increased by approximately 2-Log to a count of 5×10^7 bacteria kg^{-1} within 3 d at 20°C. At 40°C, the count increased to 8×10^7 cells kg^{-1} after just 2 d. This contrasts with the behavior of cells added to sterile seawater under similar conditions. At all temperatures, the numbers of enterococci decreased to less than 5% of the original inoculum within 4 d. Although these batch culture experiments were often extended for 2 wk, the fate of cells in sand after the initial replication phase was of less interest, being a phenomenon of the batch culture. In some experiments, the densities peaked and were maintained at this level. In other cases, usually after particularly fast growth and the attainment of high numbers, the densities peaked and decreased over the duration of the experiment (as would be expected in a batch culture).

Table 1 shows that both bacterial indicators survived well in sand across all temperatures (20–40°C) and salinities (6–40 PSU). Some interesting additional experiments were conducted, also summarized in Table 1. Nutrient enrichment to sand and seawater at 20°C and a salinity of 32 PSU had little effect on the survival of *E. coli*. When unspecified nutrients in a soil extract mixture were added to sand (as 1 mL or 20 mL of enrichment), 12 divisions of *E. coli* over 4 d and high numbers of cells were maintained thereafter. The addition of nutrients to enterococci in sand (as 1 or 20 mL of enrichment) promoted seven divisions over 4 d, and these high counts were maintained over 14 d. In seawater, growth was not enhanced (1 and 0 doublings for *E. coli* and enterococci); however, survival was enhanced because viable bacteria were maintained at the inoculum density over the 2-wk duration of the experiment.

All of the sand mesocosm experiments used wet sand (adjusted to the moisture level found in the intertidal zone). One set of experiments used dry sand (to mimic that found at the top of the beach) at a salinity of 32 PSU. Here, the same pattern of initial growth was found, regardless of the indicator bacterium. Likewise, seeding six strains of enterococci isolated directly from human fecal samples (rather than the beach isolates) into sand or water with a salinity of 32 PSU (20°C) yielded the same result: significant replication in sand and no more than one division in water.

One set of mesocosm experiments used intertidal wet sand (salinity 32 PSU; 20 or 30°C) that was collected and used without sterilization. In this case, the dramatic replication of bacteria in sand was not observed (Table 1). This was probably due to a combination of competition effects and predation; both combined to keep the indicator population in check. No replication was observed in natural seawater.

The numbers of bacteria recovered from sand by gentle washing and by vigorous shaking in PBS were compared. The cells removed by gentle washing were assumed to be living freely in the interstitial spaces between sand grains or loosely attached to the surfaces of sand particles. Cells removed by vigorous shaking were assumed to include the total number of bacteria in sand (i.e., tightly attached, free, and loosely attached bacteria). Using an independent samples t test, enterococci were found to be tightly attached to sand grains in significantly greater

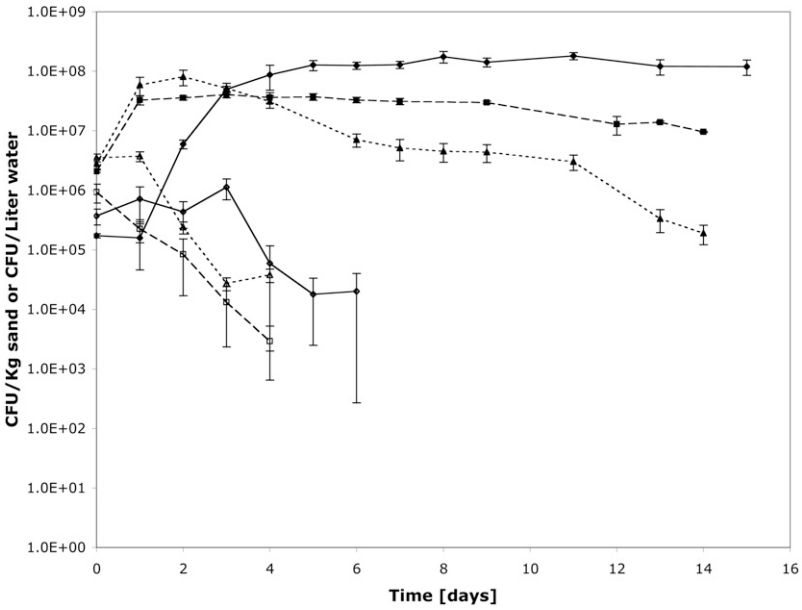

Fig. 2. Survival of enterococci in sterile beach sand and water (colony-forming units [CFU] kg^{-1} sand or CFU L^{-1} water) at different temperatures (°C) at 32 practical salinity units. Solid symbols represent sand; open symbols represent water. Shapes correspond to temperature: diamonds, 20°C; squares, 30°C; triangles, 40°C. Error bars represent 1 SE (n = 3).

numbers than loosely attached or free bacteria in wet and dry sand (wet sand: $p = 0.005$; dry sand: $p < 0.001$). Overall, in the wet sand, tightly attached enterococci were 4.1 times more common than free enterococci, whereas in dry sand 64.4 times more bacteria were tightly attached. Not surprisingly, the relative abundance of loosely attached/free enterococci (calculated as percentage of total bacteria) was significantly greater in wet sand compared with dry sand ($p = 0.001$). Numbers of tightly attached fecal coliforms significantly exceeded interstitial/free fecal coliforms (wet sand: $p = 0.02$; dry sand: $p = 0.01$), although here the factors were similar. In wet sand, 6.9 times more bacteria were attached, and in dry sand 5.4 times more bacteria were attached.

Fluorescently labeled *E. coli* were used to directly examine whether this fecal indicator was capable of attaching to sand grains in situ. After incubation with bacteria for 24 h in sterile seawater, followed by exhaustive rinsing, surface *E. coli* were observed in groups with occasional dividing cells. Counts of fluorescent *E. coli* on sand grains of different composition (i.e., silica versus $CaCO_3$) were conducted to determine if one surface was preferable for attachment and growth. Five fields of view were examined on three grains of silica and three grains of $CaCO_3$ (for a total of 15 fields of view on each grain type). After 24 h there was no significant difference in the number of attached cells between the two grain types. Silica and $CaCO_3$ grains contained an average of 2.8 and 1.3 cells per field of view, respectively. However, after Day 4 there were significantly more cells attached to the silica grains (average = 31.6 cells per field of view; SE = 5.0) compared with $CaCO_3$ sand grains (average = 12.5 cells per field of view; SE = 3.1; independent sample *t* test; $p = 0.003$). The number of cells attached to silica grains and $CaCO_3$ grains increased significantly from Day 1 to Day 4 ($p < 0.001$ and $p = 0.001$, respectively).

Sampling water in the nearshore zone (0.1–20 m) showed that numbers of *E. coli* and enterococci decreased with distance from shore (Fig. 3). In the case of *E. coli*, there was a significant decrease in the counts comparing the 0.1, 0.5, or 1.0 m counts with those at 10 or 20 m (ANOVA; $p < 0.005$). Enterococci showed a 29-fold decrease between the 0.1 and 20.0 m densities, and counts were significantly different at all distances from shore (ANOVA; $p < 0.05$).

Over 700 water samples were tested for enterococci in offshore waters between 200 and 3000 m from shore. Of these, only four samples gave counts greater than 10.0 CFU 100 mL^{-1} (the highest being 42.0 CFU 100 mL^{-1}). There was a highly significant difference ($p < 0.005$) between the mean counts in nearshore waters (13.7 ± 0. 6 SE CFU 100 mL^{-1}; $n = 546$) and the mean counts in the offshore zone (1.5 ± 0.2 SE CFU 100 mL^{-1}; $n = 717$).

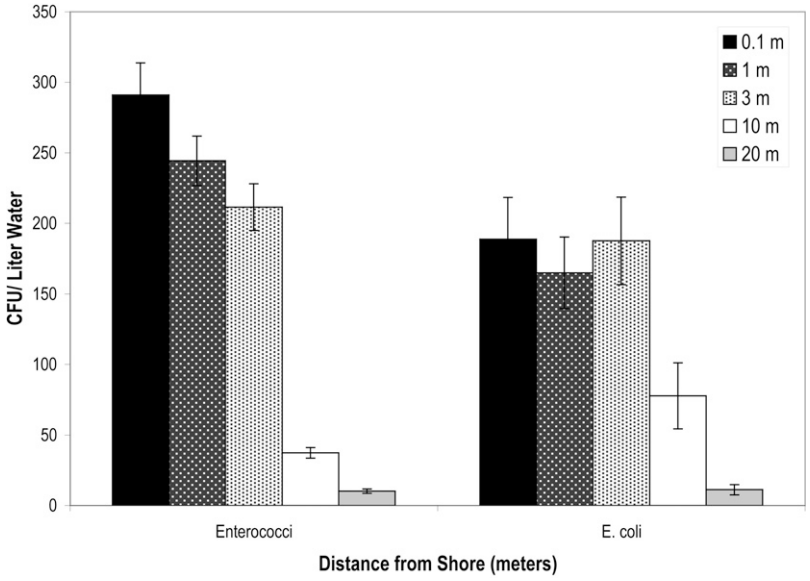

Fig. 3. Numbers of *E. coli* and enterococci (colony-forming units [CFU] L^{-1}) in water up to 20 m from shore. Error bars represent 1 SE (0.1 m, $n = 110$; 1 m, $n = 110$; 3 m, $n = 110$; 10 m, $n = 126$; 20 m, $n = 128$).

Discussion

The most striking result from the mesocosm experiments was that fecal bacteria reproduced and showed enhanced survival in beach sand relative to seawater where, in most cases, rapid die-off (or decreased viability) was evident over time. The salient message from these experiments is that *E. coli* and enterococci replicated when added to sterile sand but lost viability (rapidly or slowly depending on conditions) when added to sterile seawater. The occasional initial doubling of the population, noted in some of the water mesocosm experiments, was probably attributable to cell shock (stimulating the cell to divide) rather than true growth.

Bacteria may survive better in sand because they are protected in biofilms. Moreover, sand offers a more effective barrier to damaging UV radiation than water (Burkhardt et al., 2000). Gerba and McLeod (1976) suggested that sediments provide osmoprotectors that negate the effects of high salinities. *Escherichia coli* in muddy sediments was shown to store exogenous betaine, leading to increased salt tolerance (Pommepuy et al., 1992). Sand grains also provide a site for attachment and access to nutrients and carbon in the grain crevices (Pommepuy et al., 1992; Ashbolt et al., 1993). The addition of nutrients to sand in the present study did not increase the growth of fecal indicators, suggesting that the beach sand already contained sufficient nutrient levels to support growth and survival of enterococci and *E. coli* (Gerba and McLeod, 1976). Sand grains create a potentially favorable environment for microbial survival and growth by providing a large surface area with cracks and crevices (USEPA, 1999). In addition, surfaces can concentrate nutrients (Baty et al., 2000), allowing bacteria a nutrient-rich environment when they become attached to particle surfaces.

Previous studies have shown that benthic sediments can contain 100 to 10,000 times more fecal indicator bacteria than the overlying water column, and a few studies have shown that intertidal sand can harbor more fecal indicators than the water (Oshira and Fujioka, 1995; Craig et al., 2002; Whitman and Nevers, 2003; Bonilla et al., 2006; 2007). Hardina and Fujioka

(1991) reported *E. coli* densities between 2.2×10^3 and 2.4×10^6 colony-forming units kg^{-1} of soil along a riverbank in Hawaii, and Lipp et al. (2001) found up to 2.3×10^4 *E. coli* kg^{-1} in estuarine sediments. Apart from the recent study by the authors, there are few reports on the behavior of fecal organisms in marine beach sand, although others have reported significant counts in soil and benthic marine sediments. Concurrent to this study, several examples of increased growth of fecal bacteria in temperate freshwater and soil systems (Ishii et al., 2006; Alm et al., 2006) have added to the growing body of evidence that fecal indicator bacteria are replicating in the environment.

Because enterococci have been isolated from pristine samples of soil and water (Fujioka, 2001), it is possible that beach isolates are environmentally adapted and different in their tolerance to stressors such as salinity. However, mesocosm experiments with six fresh isolates of enterococci from human feces yielded the same trends as found for the beach isolates. These mesocosm experiments were artificial because they were set up with sterile sand or water. It would be misleading to imply that the population explosions observed in the laboratory occurred in situ. This is well illustrated in the case of the natural mesocosms containing nonsterilized sand or water with attendant bacteria and grazers. In the case of water, fecal organisms showed the expected decreased survival over time, but in the case of wet sand, the increase in indicators was not found. Presumably, any growth was kept in check by competing bacteria and/or grazing by micro-predators, particularly protozoa. These results with "natural" mesocosms do not negate the overall message that fecal bacteria survive better and remain metabolically active in beach sand than in open water. Although an increase in population abundance was not detected, it must be remembered that these "natural" mesocosms were also artificial in that the numbers of predators (such as the abundant nanoflagellates) and indigenous bacteria also increased in these closed systems. The increased competition with native bacteria and increased predation from micro-grazers probably masked increases in the fecal indicator population. Regardless, the mesocosm experiments clearly show that fecal bacteria have the potential to survive and reproduce amid beach sand, but the "natural" experiments remind us that in situ population increases on the scale found in sterile sand are unlikely.

The highest beach counts were found in the dry sand at the top of the beach (Bonilla et al., 2007), and it is likely that this was caused, in part, by in situ growth because of reduced grazing pressure. Davies et al. (1995) suggested that reduced predation in the thin water film around sand is an important factor leading to higher bacterial densities. The main regulators of bacteria, including enteric bacteria, are protozoa (Barcina et al., 1997; Rozen and Belkin, 2001), and comparative observations of micro-grazers in wet and dry sand showed that dry sand contained fewer and smaller heterotrophic protists compared with wet sand (Harz, unpublished observations).

Regardless of location on the beach, rain events (leading to storm water runoff) and the activity of people translocate bacteria across the entire beach. An example of the degree of translocation possible on a beach was shown by Bonilla et al. (2007), who reported that in high traffic areas bacterial-sized particles were moved an average of 1.6 m in just 4 h. Bacteria in wet sand are then washed out from the lower beach during tidal cycles. The high concentration of fecal bacteria (culturable cells) in waters up to 3 m from the shoreline attests to this. The rapid dropoff in enterococci density in waters from 10 to 3000 m from shore suggests that washout from the sand during tidal cycles significantly affects the shoreline area (i.e., the swash zone, 0.1–3 m). A similar effect was reported by Solo-Gabriele et al. (2000), who documented *E. coli* being washed from soil along a river bank by tidal action. In other studies, the highest bacterial counts were reported close to shore at high tide (McBride et al., 1998; Le Fevre and Lewis, 2003), and the likely source of fecal indicators into nearshore waters is the wet sand in the swash zone (Shibata et al., 2004), although on occasion storm-water runoff is an important factor. In the present study, enterococci rarely exceeded 10 cells L^{-1} offshore. This was also true in water 120 m from shore in an urban area (Le Fevre and Lewis, 2003). Because the mesocosm studies showed that sand fecal indicators survive longer and may reproduce and become "environmental," their densities in bathing water do not accurately reflect the risk from sewage-derived pathogens.

This study has shown that waters close to shore have significantly more bacteria than waters just 10 m offshore. It follows that the current practice of sampling recreational beaches in 1 m of water and 0.3 m below the surface (Clesceri, 1998) includes bacterial washout from the beach sand, thus complicating the task of water quality managers monitoring the health of beaches. These contaminants would be particularly problematic during periods of heavy wave action or wet weather events. The results also partially explain why few beach closures can be directly attributable to a pollution event. For example, in Florida, there were a total of 1745 beach closures in 2002, with 92% of the closures due to high levels of bacteria. However, only 5% were from known sewage leaks or spills (Dorfman and Stoner, 2003). The extensive sampling of offshore waters (over 700 samples) over several months failed to detect significant levels of indicator organisms, suggesting that these waters are not sources of background levels. Periodic sewage spills do occur, and it is the presence of these events that regulators are trying to detect to safeguard the health of beach users. Taking additional samples in slightly deeper water away from the influence of the beach might be a consideration for water quality managers. Here, high counts of fecal indicator organisms would likely reflect offshore plumes of sewage with greater accuracy. We recommend that studies be conducted to evaluate whether including samples 10 m from shore improve risk assessment of bathing beaches. High fecal counts in both samples might be a better predictor of true fecal contamination. A final word of caution is warranted: Assessing beach health is not straightforward, and bacteria washed from the sand might still pose a health concern depending on their source. Bonilla et al. (2007) showed that the number of bathers on a beach influenced the fecal bacterial count in the sand, probably through direct shedding, transport, and aeration phenomena. In the same study, a single gull fecal pellet increased the enterococcal count in a 3-m^2 area of beach 100- to 1000-fold in just 24 h. A thorough epidemiological study of health risks associated with exposure to indicators in beach sand is needed.

Conclusions

Mesocosm experiments were conducted to help explain the high counts of fecal bacteria observed in beach sand of three south Florida beaches (Bonilla et al., 2007). Results suggested that *E. coli* and enterococci were capable of surviving and replicating in sterile beach sand, whereas they rapidly died in seawater or became nonculturable (i.e., viable but nonculturable). Experiments investigated the numbers of bacteria in the seawater in the swash zone (0–3 m) compared with further offshore. The numbers of *E. coli* and enterococci significantly decreased with distance from shore out to 20 m. Offshore samples processed for enterococci up to 3000 m from the shore contained few cells, usually approximately 1.0 bacteria 100 mL^{-1}. These findings, combined with the mesocosm results, suggest that sand may be acting as a reservoir of fecal bacteria and that, during strong weather or tidal events, these cells may be released into the water. Implications for water managers include (i) assessing the health risks of bacteria washing out from the sand and (ii) reassessing the routine water sampling protocols if the counts of fecal bacteria are to be correlated with recent sewage contamination.

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